

IN THE CLAIMS

On page 45 of the copy of the publication, the amendments to the listing of claims serves to replace prior versions of claims from its related international application.

Listing of claims

1. (Currently Amended) A method ~~Method~~ for preparing a lyophilized matrix which, upon contact with an aqueous carrier liquid and a gas, is reconstitutable into a suspension of gas-filled microbubbles stabilized predominantly by a phospholipid, said method comprising the steps of:

- a) preparing an aqueous-organic emulsion comprising i) an aqueous medium including water, ii) an organic solvent substantially immiscible with water; iii) an emulsifying composition of amphiphilic materials comprising more than 50% by weight of a phospholipid and iv) a lyoprotecting agent;
- b) lyophilizing said emulsified mixture, to obtain a lyophilized matrix comprising said phospholipid.

Claims 2 – 40 canceled

41. (New) The method of claim 1, wherein the step a) of preparing the emulsion comprises:

- a1) preparing a suspension by dispersing the emulsifying composition and the lyoprotective agent in the aqueous medium;
- a2) admixing the obtained suspension with the organic solvent;
- a3) submitting the mixture to controlled agitation, to obtain an emulsion.

42) (New) The method of claim 1, wherein the organic solvent has a solubility in water of less than 10 g/l.

43) (New) The method of claim 42, wherein the organic solvent has a solubility in water of 0.001 g/l or lower.

44) (New) The method of claim 1, wherein the organic solvent is selected from the group consisting of branched or linear alkanes, alkenes, cyclo-alkanes, aromatic hydrocarbons, alkyl ethers, ketones, halogenated hydrocarbons, perfluorinated hydrocarbons and mixtures thereof.

45) (New) The method of claim 44, wherein the solvent is selected from the group consisting of pentane, hexane, heptane, octane, nonane, decane, 1-pentene, 2-pentene, 1-octene, cyclopentane, cyclohexane,

cyclooctane, 1-methyl-cyclohexane, benzene, toluene, ethylbenzene, 1,2-dimethylbenzene, 1,3-dimethylbenzene, di-butyl ether and di-isopropylketone, chloroform, carbon tetrachloride, 2-chloro-1-(difluoromethoxy)-1,1,2-trifluoroethane (enflurane), 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane (isoflurane), tetrachloro-1,1-difluoroethane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorononane, perfluorobenzene, perfluorodecalin, methylperfluorobutylether, methylperfluoroisobutylether, ethylperfluorobutylether, ethylperfluoroisobutylether and mixtures thereof

- 46) (New) The method of any of the preceding claims, wherein the amount of organic solvent is from about 1% to about 50% by volume with respect to the amount water.
- 47) (New) The method of claim 1, wherein the lyoprotecting agent is selected from the group consisting of carbohydrates, sugar alcohols, polyglycols and mixtures thereof.
- 48) (New) The method of claim 47, wherein the lyoprotecting agent is selected from the group consisting of glucose, galactose, fructose, sucrose, trehalose, maltose, lactose, amylose, amylopectin, cyclodextrins, dextran, inuline, soluble starch, hydroxyethyl starch (HES), erythritol, mannitol, sorbitol, polyethyleneglycols and mixtures thereof.
- 49) (New) The method of claim 1, wherein the amount of lyoprotecting agent is from about 1% to about 25% by weight with respect to the weight of water.
- 50) (New) The method of claim 1, wherein the phospholipid is selected from the group consisting of dilauroyl-phosphatidylcholine (DLPC), dimyristoyl-phosphatidylcholine (DMPC), dipalmitoyl-phosphatidylcholine (DPPC), diarachidoyl-phosphatidylcholine (DAPC), distearoyl-phosphatidylcholine (DSPC), dioleoyl-phosphatidylcholine (DOPC), 1,2 Distearoyl-sn-glycero-3-Ethylphosphocholine (Ethyl-DSPC), dipentadecanoyl-phosphatidylcholine (DPDPC), 1-myristoyl-2-palmitoyl-phosphatidylcholine (MPPC), 1-palmitoyl-2-myristoyl-phosphatidylcholine (PMPC), 1-palmitoyl-2-stearoyl-phosphatidylcholine (PSPC), 1-stearoyl-2-palmitoyl-phosphatidylcholine (SPPC),), 1-palmitoyl-2-oleylphosphatidylcholine (POPC), 1-oleyl-2-palmitoyl-phosphatidylcholine (OPPC), dilauroyl-phosphatidylglycerol (DLPG) and its alkali metal salts, diarachidoylphosphatidyl-glycerol (DAPG) and its alkali metal salts, dimyristoylphosphatidylglycerol (DMPG) and its alkali metal salts, dipalmitoylphosphatidylglycerol (DPPG) and its alkali metal salts, distearoylphosphatidylglycerol (DSPG) and its alkali metal salts, dioleoyl-phosphatidylglycerol (DOPG) and its alkali metal salts, dimyristoyl phosphatidic acid (DMPA) and its alkali metal salts, dipalmitoyl phosphatidic acid (DPPA)

and its alkali metal salts, distearoyl phosphatidic acid (DSPA), diarachidoylphosphatidic acid (DAPA) and its alkali metal salts, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), distearoyl phosphatidyl-ethanolamine (DSPE), dioleoylphosphatidyl-ethanolamine (DOPE), diarachidoylphosphatidylethanolamine (DAPE), dilinoleylphosphatidylethanolamine (DLPE), polyethyleneglycol modified dimyristoyl-phosphatidylethanolamine (DMPE-PEG), polyethyleneglycol modified dipalmitoylphosphatidylethanolamine (DPPE-PEG), polyethyleneglycol modified distearoyl phosphatidyl-ethanolamine (DSPE-PEG), polyethyleneglycol modified dioleoylphosphatidyl-ethanolamine (DOPE-PEG), polyethyleneglycol modified diarachidoylphosphatidylethanolamine (DAPE-PEG), polyethyleneglycol modified dilinoleylphosphatidylethanolamine (DLPE-PEG), dimyristoyl phosphatidylserine (DMPS), diarachidoyl phosphatidylserine (DAPS), dipalmitoyl phosphatidylserine (DPPS), distearoylphosphatidylserine (DSPS), dioleoylphosphatidylserine (DOPS), dipalmitoyl sphingomyelin (DPSP), and distearoylsphingomyelin (DSSP) and mixtures thereof.

51) (New) The method of claim 1, wherein the emulsifying composition of amphiphilic materials comprises a phospholipid or an amphiphilic material bearing an overall net charge.

52) (New) The method of claim 1, wherein the amount of phospholipid is from about 0.005% to about 1.0% by weight with respect to the total weight of the emulsified mixture.

53) (New) The method of claim 52, wherein the amount of phospholipid is from 0.01% to 1.0% by weight with respect to the total weight of the emulsified mixture.

54) (New) The method of claim 1, wherein the phospholipid includes a targeting ligand or a protective reactive group capable of reacting with a targeting ligand.

55) (New) The method of claim 1, wherein the emulsion further contains an amphiphilic material selected from the group consisting of lysolipids; fatty acids and their respective salts with alkali or alkali metals; lipids bearing polymers; lipids bearing sulfonated mono- di-, oligo- or polysaccharides; lipids with ether or ester-linked fatty acids; polymerized lipids; diacetyl phosphate; dicetyl phosphate; stearylamine; ceramides; polyoxyethylene fatty acid esters; polyoxyethylene fatty alcohols; polyoxyethylene fatty alcohol ethers; polyoxyethylated sorbitan fatty acid esters; glycerol polyethylene glycol ricinoleate; ethoxylated soybean sterols; ethoxylated castor oil; ethylene oxide (EO) and propylene oxide (PO) block copolymers; sterol esters of sugar acids; esters of sugars with aliphatic acids; esters of glycerol with (C₁₂-C₂₄) dicarboxylic fatty acids and their respective salts with alkali or

alkali-metal salts; saponins; long chain (C₁₂-C₂₄) alcohols; 6-(5-cholesten-3 β -yloxy)-1-thio- β -D-galactopyranoside; digalactosyldiglyceride; 6-(5-cholesten-3 β -yloxy)hexyl-6-amino-6-deoxy-1-thio- β -D-galactopyranoside; 6-(5-cholesten-3 β -yloxy)hexyl-6-amino-6-deoxyl-1-thio- β -D-mannopyranoside; 12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoyl]-2-aminopalmitic acid; N-succinyldioleoylphosphatidylethanolamine; 1-hexadecyl-2-palmitoylglycerophosphoethanolamine; palmitoylhomocysteine; alkylammonium salts comprising at least one (C₁₀-C₂₀) alkyl chain; tertiary or quaternary ammonium salts comprising at least one (C₁₀-C₂₀) acyl chain linked to the N-atom through a (C₃-C₆) alkylene bridge: and mixtures or combinations thereof.

56) (New) The method of claim 1, wherein the aqueous-organic emulsion of step a) is subjected to a washing step before the lyophilizing step b).

57) (New) The method of claim 1, wherein the aqueous-organic emulsion of step a) is subjected to a microfiltration step before the lyophilizing step b).

58) (New) The method of claim 1 which further comprises adding an aqueous suspension comprising a further amphiphilic compound to the aqueous-organic emulsion obtained according to step a), before the lyophilization step b), thus obtaining a second aqueous-organic emulsion comprising said further amphiphilic compound.

59) (New) The method of claim 58 which further comprises heating the mixture of said aqueous suspension and of said aqueous-organic emulsion.

60) (New) The method of claim 59, wherein said mixture is heated at a temperature of from about 40°C to about 80°C.

61) (New) The method of claim 60, wherein said amphiphilic compound is a PEG-modified phospholipid, a PEG-modified phospholipid bearing a reactive moiety or a PEG-modified phospholipid bearing a targeting ligand

62) (New) The method of claim 1, which further comprises, before the lyophilization step b), subjecting the aqueous-organic emulsion to a controlled heating.

- 63) (New) The method of claim 58 which further comprises, before the lyophilization step b), subjecting the aqueous-organic emulsion to a controlled heating.
- 64) (New) The method of any one of claims 62 or 63, wherein said controlled heating is effected at a temperature of from about 60°C to 125°C.
- 65) (New) The method of any one of claims 62 or 63, wherein said controlled heating is effected at a temperature of from about 60°C to 125°C and said emulsion is contained in a sealed vial.
- 66) (New) A method for preparing an injectable contrast agent comprising a liquid aqueous suspension of gas-filled microbubbles stabilized predominantly by a phospholipids, which comprises:
a) preparing an aqueous-organic emulsion comprising i) an aqueous medium including water, ii) an organic solvent substantially immiscible with water; iii) an emulsifying composition of amphiphilic materials comprising more than 50% by weight of a phospholipid and iv) a lyoprotecting agent; lyophilizing said emulsified mixture, to obtain a lyophilized matrix comprising said phospholipids;
b) contacting said lyophilized matrix with a biocompatible gas; and
c) reconstituting said lyophilized matrix by dissolving it in a physiologically acceptable aqueous carrier liquid, to obtain a suspension of gas-filled microbubbles stabilized predominantly by said phospholipid.
- 67) (New) The method of claim 66, wherein the biocompatible gas is selected from the group consisting of air; nitrogen; oxygen; carbon dioxide; hydrogen; nitrous oxide; inert gases; a low molecular weight hydrocarbon, including a (C₁-C₇) alkane, a (C₄-C₇) cycloalkane, a (C₂-C₇) alkene and a (C₂-C₇) alkyne; an ether; a ketone; an ester; a halogenated (C₁-C₇) hydrocarbon, ketone; ether; and a mixture of any of the foregoing.
- 68) (New) The method of claim 67, wherein the halogenated hydrocarbon gas is a perfluorinated hydrocarbon or sulfur hexafluoride.
- 69) (New) The method of claim 68, wherein the perfluorinated hydrocarbon gas is selected from the group consisting of perfluoromethane, perfluoroethane, a perfluoropropane, a perfluorobutane, a perfluoropentane, a perfluorohexane, a perfluoroheptane; perfluoropropene, a perfluorobutene, perfluorobutadiene, perfluorobut-2-yne, perfluorocyclobutane, perfluoromethylcyclobutane, a perfluorodimethylcyclobutane, a perfluorotrimethylcyclobutane, perfluorocyclopentane,

perfluoromethylcyclopentane, a perfluorodimethylcyclopentane, perfluorocyclohexane, perfluoromethylcyclohexane, perfluoromethylcyclohexane and mixtures thereof.

70) (New) An injectable aqueous suspension of microbubbles filled with a biocompatible gas and comprising a stabilizing layer predominantly comprising a phospholipid, wherein said microbubbles have a number mean diameter (D_N) of less than $1.70\ \mu\text{m}$ and a volume median diameter (D_{V50}) such that the D_{V50}/D_N ratio is of about 2.00 or lower.

71) (New) The aqueous suspension of claim 70, wherein said microbubbles have a D_N value of $1.60\ \mu\text{m}$ or lower, preferably of $1.50\ \mu\text{m}$ or lower, more preferably of $1.30\ \mu\text{m}$ or lower.

72) (New) The aqueous suspension of claim 71, wherein said microbubbles have a D_{V50}/D_N ratio of about 1.80 or lower, preferably of about 1.60 or lower, more preferably of about 1.50 or lower.

73) (New) A contrast agent for use in diagnostic imaging comprising an aqueous suspension according to any one of claims 70 to 72.

74) (New) A method for diagnostic imaging comprising administering to a subject a contrast-enhancing amount of an aqueous suspension of any one of claims 70 to 72 and imaging at least a part of said subject.

75) (New) A method for diagnostic imaging comprising administering to a subject a contrast-enhancing amount of an aqueous suspension of any one of claims 70 to 72 and imaging at least a part of said subject wherein said imaging comprises insonating said subject by means of an ultrasound device generating an ultrasound wave with a predetermined transmit frequency, from which a corresponding resonance size of microbubbles is determined, and administering a contrast agent comprising gas-filled microbubbles having a narrow size distribution and a mean size close to half the resonance size.

76) (New) The method of claim 66 wherein the step a) of preparing the emulsion comprises:

- a1) preparing a suspension by dispersing the emulsifying composition and the lyoprotective agent in the aqueous medium;
- a2) admixing the obtained suspension with the organic solvent;
- a3) submitting the mixture to controlled agitation, to obtain an emulsion.

- 77) (New) The method of claim 66, wherein the organic solvent has a solubility in water of less than 10 g/l.
- 78) (New) The method of claim 77, wherein the organic solvent has a solubility in water of less than 0.001 g/l.
- 79) (New) The method of claim 66, wherein the organic solvent is selected from the group consisting of branched or linear alkanes, alkenes, cyclo-alkanes, aromatic hydrocarbons, alkyl ethers, ketones, halogenated hydrocarbons, perfluorinated hydrocarbons and mixtures thereof.
- 80) (New) The method of claim 79, wherein the solvent is selected from the group consisting of pentane, hexane, heptane, octane, nonane, decane, 1-pentene, 2-pentene, 1-octene, cyclopentane, cyclohexane, cyclooctane, 1-methyl-cyclohexane, benzene, toluene, ethylbenzene, 1,2-dimethylbenzene, 1,3-dimethylbenzene, di-butyl ether and di-isopropylketone, chloroform, carbon tetrachloride, 2-chloro-1-(difluoromethoxy)-1,1,2-trifluoroethane (enflurane), 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane (isoflurane), tetrachloro-1,1-difluoroethane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorononane, perfluorobenzene, perfluorodecalin, methylperfluorobutylether, methylperfluoroisobutylether, ethylperfluorobutylether, ethylperfluoroisobutylether and mixtures thereof
- 81) (New) The method of claim 66, wherein the amount of organic solvent is from about 1% to about 50% by volume with respect to the amount water.
- 82) (New) The method of claim 66, wherein the lyoprotecting agent is selected from the group consisting of carbohydrates, sugar alcohols, polyglycols and mixtures thereof.
- 83) (New) The method of claim 82, wherein the lyoprotecting agent is selected from the group consisting of glucose, galactose, fructose, sucrose, trehalose, maltose, lactose, amylose, amylopectin, cyclodextrins, dextran, inuline, soluble starch, hydroxyethyl starch (HES), erythritol, mannitol, sorbitol, polyethyleneglycols and mixtures thereof.
- 84) (New) The method of claim 66, wherein the amount of lyoprotecting agent is from about 1% to about 25% by weight with respect to the weight of water.

85) (New) The method of claim 66, wherein the phospholipid is selected from the group consisting of dilauroyl-phosphatidylcholine (DLPC), dimyristoyl-phosphatidylcholine (DMPC), dipalmitoyl-phosphatidylcholine (DPPC), diarachidoyl-phosphatidylcholine (DAPC), distearoyl-phosphatidylcholine (DSPC), dioleoyl-phosphatidylcholine (DOPC), 1,2 Distearoyl-sn-glycero-3-Ethylphosphocholine (Ethyl-DSPC), dipentadecanoyl-phosphatidylcholine (DPDPC), 1-myristoyl-2-palmitoyl-phosphatidylcholine (MPPC), 1-palmitoyl-2-myristoyl-phosphatidylcholine (PMPC), 1-palmitoyl-2-stearoyl-phosphatidylcholine (PSPC), 1-stearoyl-2-palmitoyl-phosphatidylcholine (SPPC), 1-palmitoyl-2-oleylphosphatidylcholine (POPC), 1-oleyl-2-palmitoyl-phosphatidylcholine (OPPC), dilauroyl-phosphatidylglycerol (DLPG) and its alkali metal salts, diarachidoylphosphatidylglycerol (DAPG) and its alkali metal salts, dimyristoylphosphatidylglycerol (DMPG) and its alkali metal salts, dipalmitoylphosphatidylglycerol (DPPG) and its alkali metal salts, distearoylphosphatidylglycerol (DSPG) and its alkali metal salts, dioleoyl-phosphatidylglycerol (DOPG) and its alkali metal salts, dimyristoyl phosphatidic acid (DMPA) and its alkali metal salts, dipalmitoyl phosphatidic acid (DPPA) and its alkali metal salts, distearoyl phosphatidic acid (DSPA), diarachidoylphosphatidic acid (DAPA) and its alkali metal salts, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), distearoyl phosphatidyl-ethanolamine (DSPE), dioleylphosphatidyl-ethanolamine (DOPE), diarachidoylphosphatidylethanolamine (DAPE), dilinoleylphosphatidylethanolamine (DLPE), polyethyleneglycol modified dimyristoyl-phosphatidylethanolamine (DMPE-PEG), polyethyleneglycol modified dipalmitoylphosphatidylethanolamine (DPPE-PEG), polyethyleneglycol modified distearoyl phosphatidyl-ethanolamine (DSPE-PEG), polyethyleneglycol modified dioleylphosphatidyl-ethanolamine (DOPE-PEG), polyethyleneglycol modified diarachidoylphosphatidylethanolamine (DAPE-PEG), polyethyleneglycol modified dilinoleylphosphatidylethanolamine (DLPE-PEG), dimyristoyl phosphatidylserine (DMPS), diarachidoyl phosphatidylserine (DAPS), dipalmitoyl phosphatidylserine (DPPS), distearoylphosphatidylserine (DSPS), dioleoylphosphatidylserine (DOPS), dipalmitoyl sphingomyelin (DPSP), and distearoylsphingomyelin (DSSP) and mixtures thereof.

86) (New) The method of claim 66, wherein the emulsifying composition of amphiphilic materials comprises a phospholipid or an amphiphilic material bearing an overall net charge.

87) (New) The method of claim 66, wherein the amount of phospholipid is from about 0.005% to about 1.0% by weight with respect to the total weight of the emulsified mixture.

88) (New) The method of claim 87, wherein the amount of phospholipid is from 0.01% to 1.0% by weight with respect to the total weight of the emulsified mixture.

- 89) (New) The method of claim 66, wherein the phospholipid includes a targeting ligand or a protective reactive group capable of reacting with a targeting ligand.
- 90) (New) The method of claim 66, wherein the emulsion further contains an amphiphilic material selected from the group consisting of lysolipids; fatty acids and their respective salts with alkali or alkali metals; lipids bearing polymers; lipids bearing sulfonated mono- di-, oligo- or polysaccharides; lipids with ether or ester-linked fatty acids; polymerized lipids; diacetyl phosphate; dicetyl phosphate; stearylamine; ceramides; polyoxyethylene fatty acid esters; polyoxyethylene fatty alcohols; polyoxyethylene fatty alcohol ethers; polyoxyethylated sorbitan fatty acid esters; glycerol polyethylene glycol ricinoleate; ethoxylated soybean sterols; ethoxylated castor oil; ethylene oxide (EO) and propylene oxide (PO) block copolymers; sterol esters of sugar acids; esters of sugars with aliphatic acids; esters of glycerol with (C₁₂-C₂₄) dicarboxylic fatty acids and their respective salts with alkali or alkali-metal salts; saponins; long chain (C₁₂-C₂₄) alcohols; 6-(5-cholesten-3 β -yloxy)-1-thio- β -D-galactopyranoside; digalactosyldiglyceride; 6-(5-cholesten-3 β -yloxy)hexyl-6-amino-6-deoxy-1-thio- β -D-galactopyranoside; 6-(5-cholesten-3 β -yloxy)hexyl-6-amino-6-deoxy-1-thio- β -D-manno-pyranoside; 12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoyl]-2-aminopalmitic acid; N-succinyl-dioleoylphosphatidylethanolamine; 1-hexadecyl-2-palmitoylglycerophosphoethanolamine; palmitoyl-homocysteine; alkylammonium salts comprising at least one (C₁₀-C₂₀) alkyl chain; tertiary or quaternary ammonium salts comprising at least one (C₁₀-C₂₀) acyl chain linked to the N-atom through a (C₃-C₆) alkylene bridge; and mixtures or combinations thereof.
- 91) (New) The method of claim 66, wherein the aqueous-organic emulsion of step a) is subjected to a washing or microfiltration step before the lyophilizing step b).
- 92) (New) The method of claim 66 which further comprises adding an aqueous suspension comprising a further amphiphilic compound to the aqueous-organic emulsion obtained according to step a), before the lyophilization step b), thus obtaining a second aqueous-organic emulsion comprising said further amphiphilic compound.
- 93) (New) The method of claim 92 which further comprises heating the mixture of said aqueous suspension and of said aqueous-organic emulsion.

- 94) (New) The method of claim 93, wherein said mixture is heated at a temperature of from about 40°C to about 80°C.
- 95) (New) The method of claim 92, wherein said amphiphilic compound is a PEG-modified phospholipid, a PEG-modified phospholipid bearing a reactive moiety or a PEG-modified phospholipid bearing a targeting ligand.
- 96) (New) The method of claim 66 which further comprises, before the lyophilization step b), subjecting the aqueous-organic emulsion to a controlled heating.
- 97) (New) The method of claim 92 which further comprises, before the lyophilization step b), subjecting the aqueous-organic emulsion to a controlled heating.
- 98) (New) The method of any one of claims 96 or 97, wherein said controlled heating is effected at a temperature of from about 60°C to 125°C.
- 99) (New) The method of any one of claims 96 or 97, wherein said controlled heating is effected at a temperature of from about 60°C to 125°C and said emulsion is contained in a sealed vial.